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Di Fino, L.M.; D'Ambrosio, J.M.; Tejos, R.; van Wijk, R.; Lamattina, Lorenzo; Munnik, T.; Pagnussat, G.C.; Laxalt, A.M.

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ORIGINAL ARTICLE

Arabidopsis phosphatidylinositol-phospholipase C2 (PLC2) is required for female gametogenesis and embryo development

Luciano M. Di Fino[1] · Juan Martín D'Ambrosio[1] · Ricardo Tejos[2,3] · Ringo van Wijk[4] · Lorenzo Lamattina[1] · Teun Munnik[4] · Gabriela C. Pagnussat[1] · Ana M. Laxalt[1]

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Abstract

***Main conclusion AtPLC2* is an essential gene in Arabidopsis, since it is required for female gametogenesis and embryo development. AtPLC2 might play a role in cell division during embryo-sac development and early embryogenesis.**

Phosphoinositide-specific phospholipase C (PI-PLC) plays an important role in signal transduction during plant development and in the response to various biotic- and abiotic stresses. The Arabidopsis *PI-PLC* gene family is composed of nine members, named *PLC1* to *PLC9*. Here, we report that PLC2 is involved in female gametophyte development and early embryogenesis. Using two Arabidopsis allelic T-DNA insertion lines with different phenotypic penetrations, we observed both female gametophytic defects and aberrant embryos. For the *plc2-1* mutant (Ws background), no homozygous plants could be recovered in the offspring from self-pollinated plants. Nonetheless, *plc2-1* hemizygous mutants are affected in female gametogenesis, showing embryo sacs arrested at early developmental stages. Allelic hemizygous *plc2-2* mutant plants (Col-0 background) present reduced seed set and embryos arrested at the pre-globular stage with abnormal patterns of cell division. A low proportion (0.8%) of *plc2-2* homozygous mutants was found to escape lethality and showed morphological defects and disrupted megagametogenesis. *PLC2*-promoter activity was observed during early megagametogenesis, and after fertilization in the embryo proper. Immunolocalization studies in early stage embryos revealed that PLC2 is restricted to the plasma membrane. Altogether, these results establish a role for PLC2 in both reproductive- and embryo development, presumably by controlling mitosis and/or the formation of cell-division planes.

Keywords Gametogenesis · Phospholipase C · Phospholipid signaling · Megagametogenesis · Reproductive development · PLC2 immunolocalization

Abbreviations

IP_3	Inositol 1,4,5-trisphosphate
PA	Phosphatidic acid
PIP	Phosphatidylinositol phosphate
PI-PLC	Phosphoinositide-specific phospholipase C
PIP_2	Phosphatidylinositol 4,5-bisphosphate
Ws	Wassilewskija

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✉ Gabriela C. Pagnussat
gpagnussat@mdp.edu.ar

✉ Ana M. Laxalt
amlaxalt@mdp.edu.ar

[1] Instituto de Investigaciones Biológicas IIB-Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Mar del Plata, 7600 Mar del Plata, Argentina

[2] Facultad de Recursos Naturales Renovables, Universidad Arturo Prat, 111093 Iquique, Chile

[3] Centro de Biología Molecular Vegetal, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, 7800003 Santiago, Chile

[4] Swammerdam Institute for Life Sciences, Section Plant Cell Biology, University of Amsterdam, 1098 XH Amsterdam, The Netherlands

Introduction

Mammalian phosphoinositide-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the signaling products diacylglycerol (DAG) in the membrane and the water-soluble inositol 1,4,5-trisphosphate (IP_3). DAG activates members of the protein kinase C (PKC) family, and IP_3 activates ligand-gated calcium channel releasing Ca^{2+} from intracellular stores. In plants, the PI-PLC signaling is less clear, since they lack homologues of PKC and IP_3 receptors (Munnik 2014). Yeast also lacks the prime targets of the mammalian PI-PLC pathway; however, the sole PLC protein has been implicated in nutrient sensing, filamentous growth, actin organization, transcriptional regulation, mRNA export, vacuolar fusion, and stress response (Rebecchi and Pentyala 2000; DeLillo et al. 2003; York 2006). In addition, yeast PLC affects kinetochore function, altering chromosome segregation during mitosis (Desai et al. 2009).

How PLC functions in yeast and plant cells are still not clear, but there is evidence that the phosphorylated products of IP_3 and DAG have a role in cell signaling, including various inositol polyphosphates (IPPs), phosphatidic acid (PA), and diacylglycerol pyrophosphate (DGPP) (Munnik and Vermeer 2010; Testerink and Munnik 2011). In plants, PA recruits and regulates several protein targets (Arisz et al. 2009; Testerink and Munnik 2011). IPPs might act on releasing Ca^{2+} to the cytoplasm by regulating ion channels, but might also affect gene transcription or mRNA export (Zonia and Munnik 2006; Lee et al. 2015). Another role of IPPs during plant signaling is the recent discovery of IP_6 in the crystal structure of the auxin receptor, TIR1, and of IP_5 in the jasmonate receptor, COI1 suggesting their involvement in hormone perception and signaling (Tan et al. 2007; Sheard et al. 2010). Alternatively, PLC could attenuate signaling functions of the PI-PLC substrates PIP and PIP_2, which are emerging lipid second messengers themselves (Munnik and Nielsen 2011).

Nonetheless, PI-PLCs have been implicated in various plant signaling processes, especially related to biotic- and abiotic stresses (Munnik 2014). Increase in PI-PLC activity has been reported in response to drought and salt stress, ABA treatment, as well as during biotic interactions (Munnik 2014; Hong et al. 2016). Besides stress responses, PI-PLCs play an important role in pollen-tube growth (Monteiro et al. 2005; Dowd et al. 2006; Helling et al. 2006) and meanwhile we were conducting our experiments, an Arabidopsis PI-PLC was implicated in gametogenesis (Li et al. 2015).

The *Arabidopsis thaliana* genome contains nine PI-PLC-coding genes, named *AtPLC1* to *AtPLC9* (Mueller-Roeber and Pical 2002). While AtPLC3 and AtPLC9 have been implicated in heat stress signaling (Gao et al. 2014), at the time we initiated our research, no role for AtPLC2 was known yet. To start our research, two T-DNA insertion mutant lines were obtained, one with a Ws background (*plc2-1*) and the other with a Col-0 background (*plc2-2*). For *plc2-1* mutant allele, no homozygous mutants were recovered in the offspring from self-pollinated hemizygous mutant plants. Instead, an embryo-sac-development arrest was found, indicating that PLC2 is essential for early female gametophyte development. Using an independent insertion allele in the Col-0 background, *plc2-2,* we again found that disruption of *PLC2* led to sterility, similar to what was recently observed by Li et al. (2015). Our results presented here, together with those of Li et al. (2015), show that the *plc2-2* displayed defects in both female and male gametophytes, and also during early embryogenesis, rendering mainly hemizygous plants. These results are in contrast to a recent study by Kanehara et al. (2015), where *plc2-1* homozygous mutant plants were obtained and maintained throughout different generations, suggesting that knock-out plants have normal gametophyte and embryo development. The results of the present study suggest that PLC2 plays an essential role during reproductive- and embryonic development, presumably by regulating mitosis and/or the formation of the cell-division plane. These and other roles for PLC will be discussed.

Materials and methods

Plant material and growth conditions

T-DNA insertion lines for the *AtPLC2* gene (At3g08510): *plc2-1* insertion line (FLAG 506C04) in Wassilewskija (Ws) ecotype from Institut National de la Recherche Agronomique (INRA); *plc2-2* insertion line (SALK_152284) in Columbia-0 (Col-0) ecotype from Ohio State University Arabidopsis Biological Resources Center (ABRC). Surface sterilized seeds were plated on Murashige and Skoog plates with 50 µg/mL kanamycin and kept at 4 °C for 2 days. Then, they were grown at 25 °C using a 16-h photoperiod. Resistant seedlings (green seedlings with true leaves) were then transferred onto soil (soil:vermiculite:perlite, 3:1:1, by weight) and grown under the conditions described. T3 homozygous *pPLC2::PLC2-GFP* lines were generated by transforming *plc2-1/PLC2* plants with the plasmid pMDC 107 containing the complete *AtPLC2* gene (−1500 bp until the codon before of the STOP codon, see supporting information Fig. S1) followed by the GFP coding gene. They were selected on MS (Murashige and Skoog) medium supplemented with 10 µg/mL

hygromycin and 50 µg/mL kanamycin and resistant seedlings were then transferred to soil and grown under the conditions described above. Transgenic lines harboring *pPLC2::PLC2-GFP* with *plc2-1/plc2-1* background in T2 generation were selected for further characterization.

Molecular characterization of insertional lines

Genomic DNA was extracted from leaves of 14-day-old plants, as described in Pagnussat et al. (2007). The characterization of the *plc2-1* and *plc2-1* lines was performed by PCR using specific T-DNA and *AtPLC2* primers, as indicated in Fig. S1. For the complementation line, to distinguish between the endogenous from the trans-*PLC2* gene, we use a 3′UTR genomic sequence specific primer. All primers used are listed in supporting information Table S1.

Segregation analysis line

For self-cross analysis, hemizygous plants were allowed to self-pollinate and progeny seed was collected. Reciprocal crosses were performed, as described previously (Pagnussat et al. 2005). F1 seed was germinated on MS medium containing 50 µg/mL kanamycin and the numbers of resistant- and sensitive plants were scored.

Microscopic analysis

For female gametophyte observations, flowers from different developmental stages with at least 40 ovules per pistil were dissected and cleared for 4–5 h in Hoyers solution (Pagnussat et al. 2007). For embryo observations, young siliques of 4–5 days were dissected and then cleared overnight in Hoyers solution. The material was observed on a Zeiss Axioplan Imaging 2 microscope equipped with DIC optics. Images were captured on an Axiocam HRC CCD camera (Zeiss) using the Axiovision program (version 4.2).

ß-Glucuronidase (GUS) expression

For GUS staining, developing carpels and siliques was dissected and incubated in GUS-staining buffer [5 mM EDTA, 0.1% Triton X-100, 5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, and 1 mg/mL X-Gluc (Rose Scientific) in 50 mM NaPi buffer, pH 7.0] for 2–6 h at 37 °C. The material was observed on a Zeiss Axioplan imaging 2 microscope under DIC optics. Images were captured on an Axiocam HRC CCD camera (Zeiss) using the Axiovision program (version 4.2).

Immunolocalization and Western-blot analysis

Polyclonal antibodies were prepared, as described in Otterhag et al. (2001). A peptide KDLGDEEVWGREVPSFIQR corresponding to residues 266–284 of AtPLC2 was synthesized. One rabbit was immunized at a 2-week interval and serum collected after the second boost.

PLC2 immunolocalization in embryos was performed, as described by (Sauer et al. 2006). The PLC2 antibodies were used in 1:2000 dilutions. The secondary anti-rabbit antibody coupled to Cy3 (Sigma-Aldrich) was diluted 1:600. DAPI (1 mg/mL) was included in the mounting solution to visualize the nuclei. Embryos were observed using confocal microscopy (Nikon Eclipse C1 Plus confocal microscope) using the EZ-C1 3.80 imaging software and Ti-Control. For Cy3 red, the 408-nm line of the confocal laser was used for excitation and the red photomultiplier channel of the confocal microscope was used for image acquisition.

Protein extraction buffer [100 mM NaPi pH 7.5, 150 mM NaCl, 1 mM EDTA and Sigma proteinase inhibitor cocktail] was added to an equal volume of ground tissue from flowers, vortexed, and centrifuged for 10 min at 10,000*g*. Protein concentration in the supernatant was determined. Samples were loaded onto a 10% SDS–polyacrylamide gel, blotted on nitrocellulose, and stained with Ponceau S for loading control. Membranes were incubated overnight in PBST containing polyclonal anti-PLC2 antibody (1:2000). The blot was washed three times with PBST and the PLC2 protein revealed by anti-rabbit IgG antibody coupled to alkaline phosphatase according to manufacture's instructions (Sigma). The anti-PLC2 antibody recognizes a specific band of approximately 66 kDa, which corresponds to the predicted molecular weight of AtPLC2. Western-blot analysis using recombinant PLCs or plant extracts showed that the antibody only recognize PLC2 and not the other PLCs (data not shown). At longer developer-incubations, other non-specific bands of different molecular weights appear in total plant extracts.

RT-PCR

Total RNA was extracted by the Trizol method according to the instructions of the manufacturer (Invitrogen). Complementary DNA (cDNA) was synthesized on 2 µg of total RNA by MMLV reverse transcriptase (RT) from Promega using oligo-dT primer. cDNA was used for RT-PCR with the primers indicated in the supporting information Fig. S1 and Table S1. PCR products were analyzed by electrophoresis on agarose gels and visualized with SYBRSafe-DNA Gel Stain (Invitrogen).

Pollen assays

For in vivo pollen germination assay, aniline blue staining was performed in pre-emasculated mature wild-type flowers, pollinated with either wild-type- or *plc2-2/plc2-2* pollen. The pollinated pistils were collected 3 or 24 h after pollination and stained, as described by Mori et al. (2006). The stained pistils were observed using a Zeiss Axioplan imaging 2 microscope. Images were captured on an Axiocam HRC CCD camera (Zeiss) using the Axiovision program (version 4.2).

For in vitro pollen germination assay, pollen grains were collected from wild types, *PLC2/plc2-2* and *plc2-2/plc2-2* open flowers, incubated in germination medium (0.01% (w/v) boric acid, 5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgSO_4$, 10% (w/v) sucrose, 0.8% (w/v) agarose, and pH 7.5) at 22 °C for 16 h and observed on an Eclipse 2000 Nikon microscope.

To examine pollen viability, flower buds prior to opening were harvested, pollen grains were treated with Alexander staining (Alexander 1969) for 1 h at 37 °C and then observed on an Eclipse 2000 Nikon microscope.

To examine pollen cellular morphology, DAPI staining was performed in mature pollen grains. Pollen harvested from open flowers was treated with DAPI solution (final concentration of 600 nM dissolved in McIlvaine's buffer pH 7.0) for 5 min and then observed visualized by fluorescence microscopy with an excitation filter of 358 nm and emission filter of 461 nm in an Eclipse E 200 (Nikon, Tokyo, Japan) microscope.

Lipid analysis

Leaf discs (5 mm Ø) or flowers (three per tube) were labeled 5 h by incubation with 370000 Bq carrier-free $32PO_4$ in 100 µL 2.5 mM Mes buffer, pH 5.7 (KOH), and 1 mM KCl. Labeling was stopped by adding 5% (v/v) perchloric acid for 5–10 min. Lipids were subsequently extracted, separated by thin layer chromatography, and quantified by orthophosphate imaging (Munnik and Zarza 2013).

Accession numbers

At3g08510, *AtPLC2* gene. FLAG 506C04, *plc2-1* insertion line in Wassilewskija (Ws) ecotype. SALK_152284, *plc2-2* insertion line in Columbia-0 (Col-0) ecotype.

Results

Characterization of the *plc2-1* T-DNA insertion line

To study the role of PLC2, an Arabidopsis T-DNA insertion line in the Wassilewskija (Ws) genetic background was obtained, which contained a T-DNA insertion at the end of the first intron (Fig. S1). Searching for homozygous mutants, we analyzed the progeny of self-pollinated normal-looking hemizygous *plc2-1/PLC2* plants by PCR (Fig. S1). To our surprise, from kanamycin-resistant seedlings, we did not recover homozygous plants ($n = 130$), which suggested defects either during gametophyte development or during embryogenesis. Furthermore, when the transmission of the T-DNA was studied by following the kanamycin-resistant phenotype (K^r) in the offspring of self-pollinated *plc2-1/PLC2* plants, it did not show the 3:1 (K^r:K^s) ratio expected for the segregation of a single Mendelian gene (Table 1, Chi-square $P < 0.0001$). Instead, the kanamycin-resistant/sensitive phenotype ratio was approximately 1.5:1 (K^r:K^s, $n = 1067$), which is close to the expected ratio when the mutation affects an essential gene for the development of one of the gametophytes (Pagnussat et al. 2005). When siliques of *plc2-1/PLC2* plants were opened and analyzed, around 45% of aborted ovules were found (Fig. 1). To further investigate whether the abortion ratio observed in *plc2-1/PLC2* mutants was the

Table 1 T-DNA transmission analysis

	K^r	K^s	Segregation ratio (K^r:K^s)
Self pollinitation			
plc2-1/PLC2 × *plc2-1/PLC2*	642	425	1.51:1*[1]
Manual pollinitation			
♂ × ♀			
PLC2/PLC2 × plc2-1/PLC2	102	192	0.53:1*[2]
plc2-1/PLC2 × *PLC2/PLC2*	125	132	0.94:1

Seeds resulting from the indicated crosses were collected and grown on selective plates containing 50 mg/L kanamycin. Seedlings were scored as resistant (K^r) or sensitive (K^s) and the segregation ratio was calculated

*[1] Significantly different from the expected 3:1 (K^r:K^s) ratio for a mutation in a non-essential gene (Chi-Square $P < 0.0001$)

*[2] Significantly different from the expected 1:1 (K^r:K^s) ratio for a gene not required in female garnetophyte development/function (Chi-Square $P < 0.0001$)

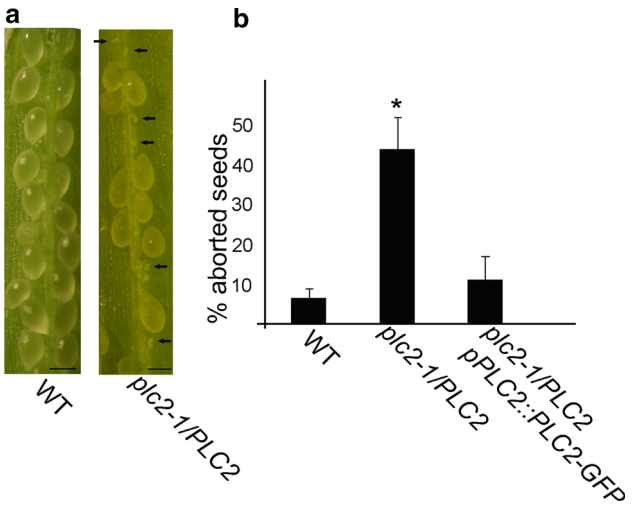

Fig. 1 *plc2-1/PLC2* mutants have reduced seed sets. **a** Dissected siliques obtained from a wild-type plant showing viable seeds (*left panel*) and from *plc2-1/PLC2* plant at a comparable developmental stage (*right panel*), where arrows indicate aborted ovules. *Scale bars* 0.5 mm. **b** Percentage of aborted seeds quantified in siliques from wild-type ($n = 1590$), *plc2-1/PLC2* ($n = 1328$) and *plc2-1/PLC2* complemented plants with the PLC2 gene (*pPLC2::PLC2-GFP*) ($n = 725$). The data represent mean ± SE. The *asterisk* indicates statistically significant differences compared to the wild type (*t* test $P < 0.01$)

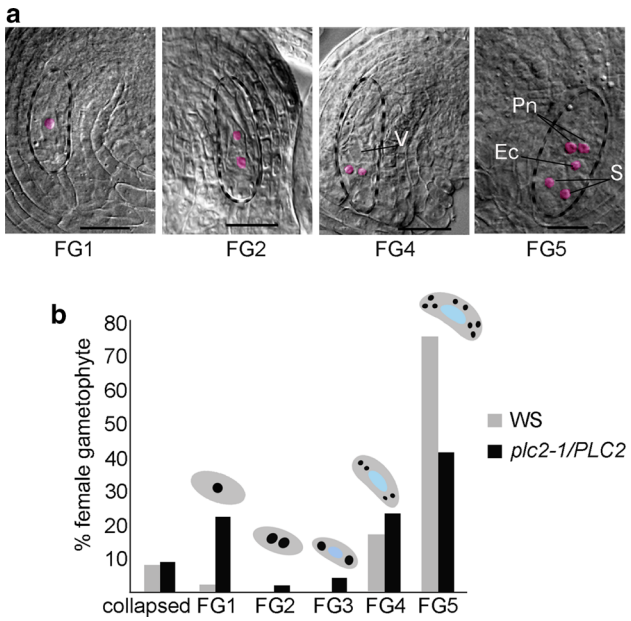

Fig. 2 Female gametogenesis is impaired in *plc2-1/PLC2* mutants. **a** Ovules at different developmental stages obtained from *plc2-1/PLC2* pistils of a size between 1.5 and 2 mm. Pistils were dissected under a binocular microscope and the ovules mounted and cleared in Hoyer's solution. Images were obtained using a DIC-equipped microscope. The boundaries of the developing embryo sacs (*dashed lines*) and their nuclei (*magenta*) are indicated. *Pn* polar nuclei, *EC* egg cell, *S* synergid cell, *V* vacule. *Bars* 25 µm. **b** Percentage of female gametophyte arrested at different stages of embryo sac development in *plc2-1/PLC2* and wild type pistils

result of defects in either the male and/or the female gametophyte, we crossed hemizygous plants as males or females with WT plants, and studied the transmission of the kanamycin-resistant phenotype. Table 1 shows that the *plc2-1* mutant allele does not affect the transmission through the male gametophyte, as the ratio obtained was close to the expected 1:1 (K^r:K^s = 0.94:1). However, only approximately 25% of the offspring showed a resistant phenotype when hemizygous pistils were pollinated with WT pollen, suggesting that *PLC2* is an essential gene required for female gametophyte development or function (Table 1, K^r:K^s = 0.53:1 from 1:1 expected, Chi-square $P < 0.0001$). Complementation studies of *plc2-1/PLC2* plants transformed with the *PLC2* gene (*pPLC2::PLC2-GFP*) reestablished the WT phenotype (Fig. 1), further demonstrating that the observed defect is due to the T-DNA insertion in the *PLC2* gene.

To characterize the basis of the defect observed in *plc2-1* transmission, we studied the terminal phenotype of female gametophytes from emasculated *plc2-1/PLC2* flowers. The WT female gametophyte structure originates from an haploid megaspore or functional megaspore (FM), which is the surviving spore from the original four haploid cells produced after female meiosis in the nucellus of the ovule (stage FG1; Fig. S2; Drews and Koltunow 2011). Three successive mitotic nuclear division cycles originate a syncytium of eight nuclei (stages FG2–FG5; Fig. S2). Further differentiation gives rise to the seven cells that comprise the mature embryo sac (stages FG6 and FG7; Fig. S2). When *plc2-1/PLC2* pistils from emasculated flowers were opened and the developing ovules analyzed, we found around 50% of the ovules with collapsed embryo sacs. To narrow down the stage at which the gametophyte development was arrested, we analyzed WT and *plc2-1/PLC2* pistils of a size between 1.5 and 2 mm, where gametophytes should be at the FG5 stage (Cigliano et al. 2013). As shown in Fig. 2, approximately 30% of the ovules presented embryo sacs arrested at FG1–FG2 stages and 20% at FG4 stage, while the rest of the female gametophytes reached an FG5 stage ($n = 234$). Analysis of WT pistils at the same developmental point revealed that from a total of 181 gametophytes studied, 91.8% of them were at FG4–FG5, 1.6% were at FG1 stage, while 7.7% were collapsed (Fig. 2). These results suggest that the mutation in *PLC2* affects the mitotic events of the female gametophyte development.

PLC2 expression pattern and subcellular localization

To analyze the expression of *PLC2* at different stages of flower development, transgenic plants carrying the *PLC2*-putative promoter sequence driving the expression

of the GUS-coding gene (*pPLC2*::GUS fusions) were studied (Fig. 3). *pPLC2*::GUS revealed GUS activity within the embryo sac only at FG1–FG2 stages, whereas no GUS staining was detected between FG3 and FG7 stages. After fertilization, GUS expression was observed outside the zygote, where the synergid cells undergo degeneration, triggered by pollination (Fig. 3). GUS expression was also observed in the maternal tissues of the ovule, at the chalazal pole, and in the embryo at dermatogen and late globular stage. No GUS expression was detected at later stages of embryogenesis. Immunolocalization studies using an anti-PLC2 antibody showed that PLC2 is localized at the plasma membrane in the embryo proper cells in late globular stage (Fig. 3), suggesting that in addition to its role during megagametogenesis, PLC2 may also have a function after fertilization during embryogenesis.

Characterization of a Col-0, *plc2-2* T-DNA insertion line

As no other mutant alleles for the *AtPLC2* gene were found in the WS background, an independent T-DNA insertion line in the Col-0 ecotype was studied (*plc2-2;* Fig. S1). *plc2-2* carries a T-DNA insertion in the middle of the sixth exon. Surprisingly, siliques of *plc2-2/PLC2* plants only showed approximately 25% of aborted seeds ($n = 984$), almost half of the amount found in *plc2-1/PLC2* plants. Following the transmission of the T-DNA analyzing the kanamycin-resistant phenotype in the offspring of self-

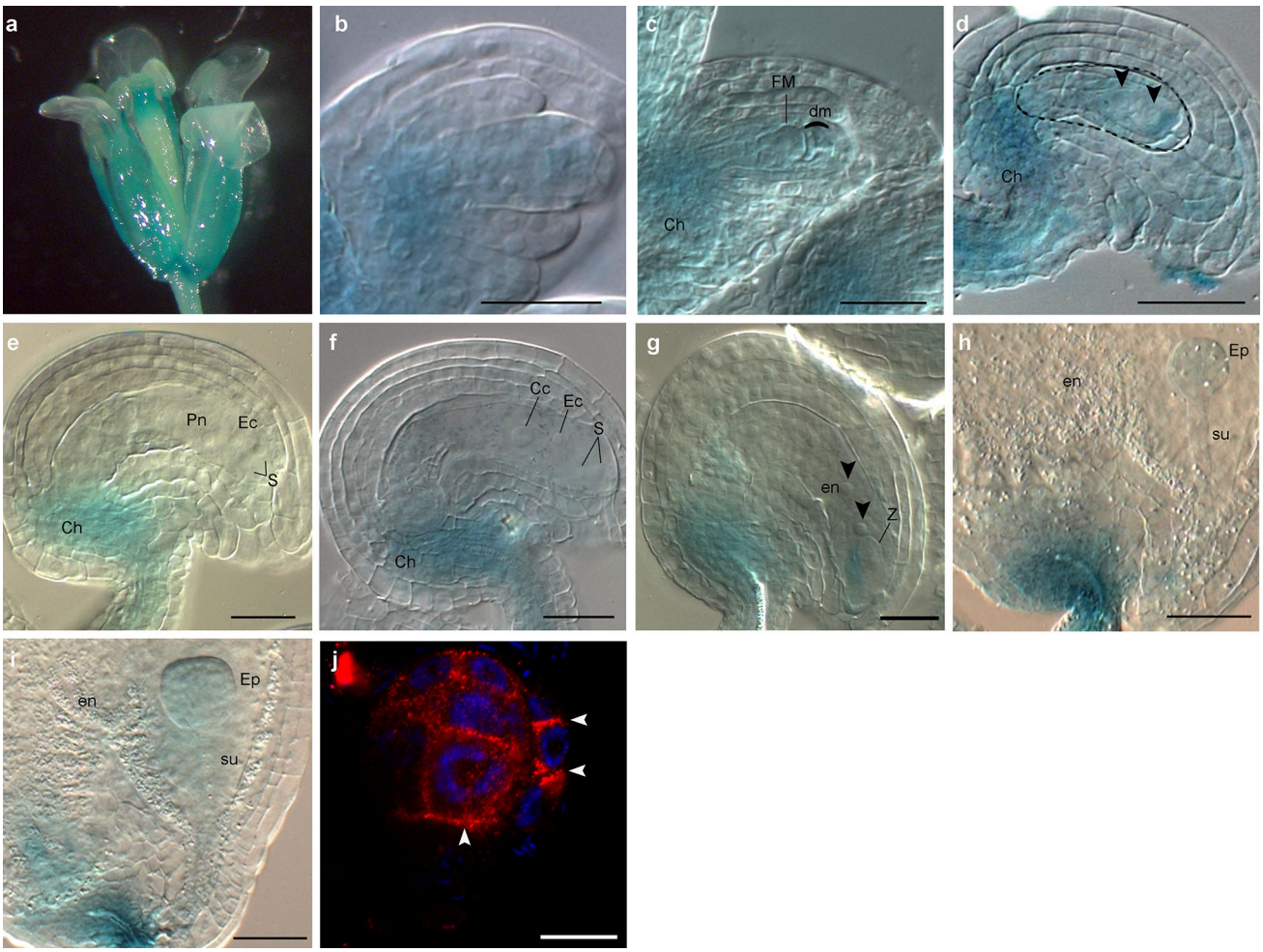

Fig. 3 Expression pattern of PLC2 in megagametogenesis and early embryo development. *PLC2* promoter (*pPLC2*) transcriptional reporter visualization at different stages of gametophyte development and embryogenesis. GUS activity was examined in **a** flowers, **b** megasporocyte, **c** FG1 stage showing the functional megaspore (FM) and degenerated megaspores (dm), **d** FG2 stage showing two nuclei (*arrowheads*), **e** FG5 stage, **f** FG7 stage, **g** zygote (z) showing endosperm nuclei (*arrowheads*), **h** embryo at dermatogen stage and **i** embryo at late globular stage. In **d,** the embryo sac is outlined in black. **j** Embryo immunolocalization using anti-AtPLC2 specific antibodies and imaged using confocal microscopy. *Arrowheads* highlight the AtPLC2 signals. Nuclei are stained with DAPI. *FM* functional megaspore, *dm* degenerated megaspore, *ch* chalaza, *Pn* polar nuclei, *Ec* egg cell, *S* synergid cells, *Cc* central cell, *en* endosperm, *Z* zygote, *Ep* embryo proper, *su* suspensor. **a–i** *Bars* 25 μm, **j** 10 μm

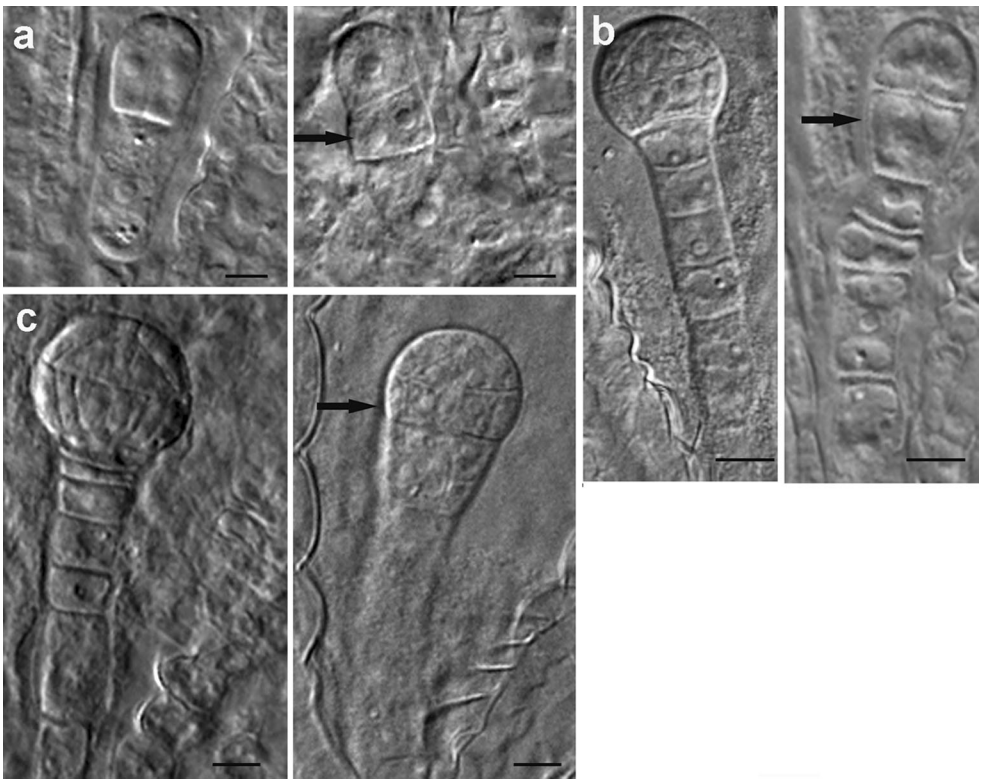

Fig. 4 Embryo development is impaired in *plc2-2* mutants. **a** 2/4-celled, **b** dermatogen, and **c** early globular stages of embryo development in siliques of self-pollinated *plc2-2/PLC2* plants. Representative pictures of normal embryos (78%) are shown on the *left panels*, while examples of embryos obtained from the same silique with abnormal cell division planes (22%) are shown on the *right panel*. *Arrows* indicate aberrant cell division planes. *Bars* 10 μm

pollinated hemizygous plants, a ratio close to 2:1 (K^r:K^s) was found (Table S2), which is close to the ratio expected for a recessive mutation that affects an essential gene involved in embryo development (1.84:1, $n = 1481$, Chi-square $P = 0.1367$). Thus, embryo morphology was observed during different development stages of siliques from self-pollinated *plc2-2/PLC2* plants. Around 25% of the embryos show abnormal cell-division planes during early stages of development, ranging from 2/4 cells to dermatogen and early globular stages ($n = 251$, Fig. 4).

When the offspring of *plc2-2/PLC2* self-pollinated plants was analyzed, a small proportion of the seedlings showed a reduced size and presented a delay in the emergence of true leaves on kanamycin MS media (0.8%, $n = 490$; Fig. 5). These plants were found to be homozygous for the T-DNA insertion by PCR-based genotyping (Fig. 5). Neither *PLC2* transcripts nor PLC2 protein was detected in these plants (Fig. 5). While hemizygous mutant plants did not show any sporophytic phenotype compared to WT plants, *plc2-2/plc2-2* plants were smaller (Fig. 5) and contained short siliques that did not show any developing ovules or seeds (Fig. 5). The flowers presented short stamens (Fig. 6). Individual female gametophytes in *plc2-2/plc2-2* pistils appeared to be filled with nucellar cells, suggesting that an early sporophytic defect might be preventing normal embryo-sac development in the homozygous mutants (Fig. 6). WT and knock-out mutants showed no significant differences in polyphosphoinositols and PA measured by labeling the phospholipids with $^{32}P_i$ in leaf discs or flowers (Fig. S3).

In a parallel study, Li et al. (2015) have recently suggested a role for *plc2-2/plc2-2* mutant in male fertility. As homozygous *plc2-2* defects in the sporophytic tissues might affect gametophyte development or function, we performed our studies using *plc2-2/PLC2* hemizygous mutant plants. Thus, we performed *plc2-2/PLC2* backcrosses to address if PLC2 has a function in pollen as well. We found that *plc2-2/PLC2* showed defects in the transmission through the female gametophyte (segregation ratio of resistant phenotype in the progeny of reciprocal crosses was significantly different from 1:1, Chi-square $P = 0.0253$; Table S2), as for the *plc2-1* allele (Table 1). In addition, *plc2-2/PLC2* mutants were also affected in the transmission through the male gametophyte (Chi-square $P < 0.0001$; Table S2). We then examined pollen morphology and germination to study the basis of this abnormal transmission ratio. Mature pollen grains of both *plc2-/PLC2* and *plc2-2/plc2-2* mutant plants have regular tri-

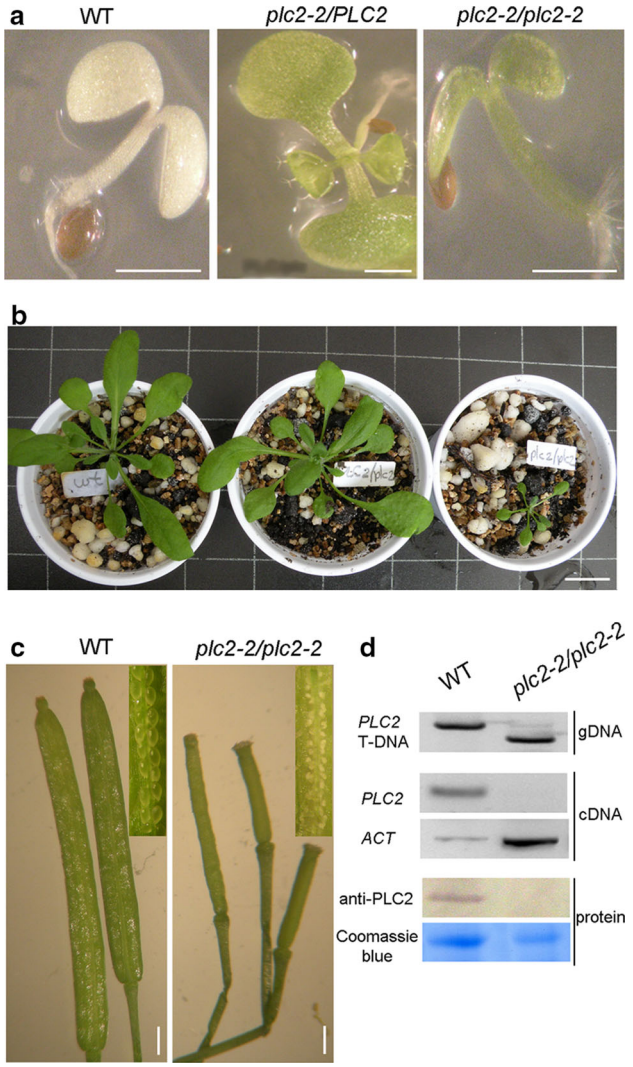

Fig. 5 Characterization of *plc2-2* homozygous mutants that escape embryonic lethality. **a** Seeds of self-pollinated *plc2-2/PLC2* plants were germinated in MS medium containing 0.1 mg/L kanamycin for 7 days. Note that WT seedlings are kanamycin sensitive. *Bars* 1 mm. **b** Three-week-old kanamycin resistant plants. In the case of WT, seeds were grown for 7 days in absence of antibiotic and then transferred to soil. *Bar* 2 cm. **c** Phenotype of siliques obtained from WT (*left panel*) and *plc2-2/plc2-2* plants (*right panel*). Insets correspond to dissected siliques showing viable seeds in WT and aborted ovules in *plc2-2/plc2-2* plants. *Bars* 1 mm. **d** Molecular characterization of *plc2-2* homozygous mutants. Genotyping of *plc2-2* plants using *PLC2*- and T-DNA border-specific primers. *PLC2* expression analysis by RT-PCR using RNA extracted from WT and *plc2-2* pistils. Western-blot analysis using protein extracted from WT and *plc2-2* flowers and an antiPLC2 antibody

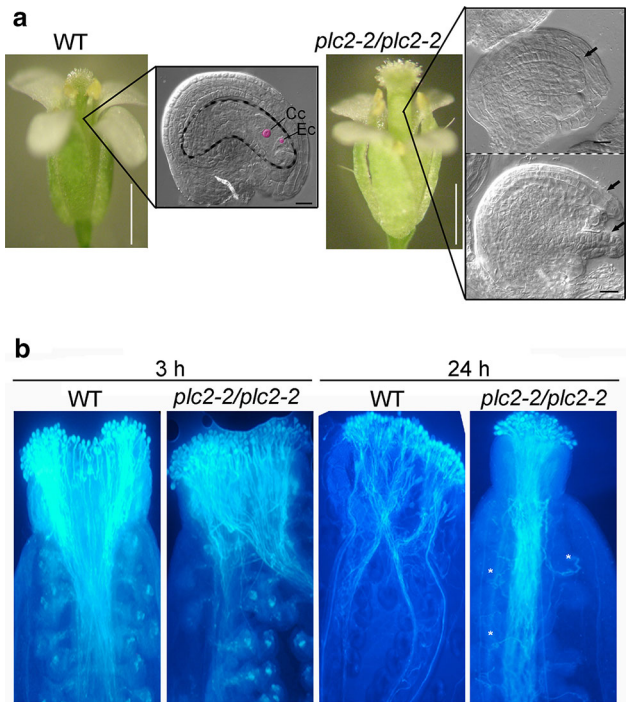

Fig. 6 Gametophyte analysis of *plc2-2* homozygous mutants. **a** Flowers and ovules obtained from WT and *plc2-2* homozygous mutant. Arrows indicate abnormal development of integuments. No embryo sac is distinguishable in *plc2-2* ovules. *Cc* central cell, *Ec* egg cell. *Bars* (flower) = 1 mm, *bars* (ovules) = 10 µm. **b** Pollen tube growth in WT pistils pollinated with WT or *plc2-2* pollen. Flowers were emasculated and pistils were hand pollinated. Three and 24 h after pollination pistils were cut, stained with aniline blue and observed by fluorescence microscopy

cellular structures and similar viability compared to those of wild-type plants (Fig. S4). However, lower germination rates were obtained for pollen from *plc2-/PLC2* plants as compared to WT, while pollen obtained from *plc2-2/plc2-2* mutant plants does not germinate in vitro (Fig. S4). Based on the fact that we were able to obtain *plc2-2/plc2-2* homozygous plants, we speculate that at least a low proportion of knock-out pollen grains should be able to germinate *in planta*. To study that possibility, pollen grains obtained from *plc2-2* homozygous mutant plants were used to fertilize emasculated WT pistils and the growth of pollen tubes was followed by aniline blue staining. As shown in Fig. 6, pollen grains from *plc2-2/plc2-2* plants are able to germinate in emasculated WT pistils and to fertilize WT ovules. Accordingly, kanamycin-resistant seedlings were obtained from this cross. Altogether, these results confirm the male gametophytic defects reported by Li et al. (2015) and add that *PLC2* is essential for the female gametophyte too, and is crucial for embryo development.

Discussion

In the last years, attention was directed to elucidate the role of different PI-PLCs. Arabidopsis PI-PLC genes are transcriptionally induced by various stresses (Hunt et al. 2004; Tasma et al. 2008). Using reverse genetics, the role of a few members of this family has been related to specific stresses. For instance, ABA induces the expression of *PLC1*, and *PLC1* was required for the induction of ABA-

responsive genes (Sanchez and Chua 2001). Knock-out mutants of Arabidopsis *PLC3* and *PLC9* showed a role for these PLCs in thermotolerance (Zheng et al. 2012; Gao et al. 2014). Silencing of tomato *SlPLC2*, *SlPLC4,* and *SlPLC6* showed that different PLC isoforms have a distinct function in plant immune response (Vossen et al. 2010; Gonorazky et al. 2014, 2016). *AtPLC2* is constitutively and highly expressed compared to the other members of the family (Hirayama et al. 1997; Hunt et al. 2004; Tasma et al. 2008). AtPLC2 is a plasma-membrane protein that is ubiquitously expressed and specifically phosphorylated upon flagellin and sucrose treatments (Niittyla et al. 2007; Nuhse et al. 2007). In this work, we show that *AtPLC2* is expressed at early developmental stages of female gametophyte development, during early embryogenesis and in the maternal tissues of the ovule, at the chalazal pole. Seeking for knock-out mutant plants for *AtPLC2*, we found that PLC2 is necessary for normal development. PLC2 has a role in Arabidopsis female gametophyte development, where it seems to be required for normal progression of the mitotic divisions that take place from FG1 to FG4 stages. Accordingly, GUS expression studies showed that *PLC2* is tightly regulated during female gametophyte development. *PLC2* is expressed within the embryo sac only at FG1-FG2 stages. This correlates with around 45% of embryo sacs being arrested at FG1 in *plc2-1/PLC2* hemizygous plants. Thus, PLC2 seems to be required gametophytically for mitotic progression, as hemizygous mutants show aberrant and arrested embryo sacs.

AtPLC2 was also expressed during the first stages of embryogenesis. PLC2 immunolocalization studies showed expression in pre-globular and globular stages during embryo development. This expression pattern together with the *plc2-2* phenotype suggests that in addition to its role during megagametogenesis, PLC2 might also have a function after fertilization, where seems to be important to regulate cell-division planes. It has been shown that the abundance of PI4P and PI(4,5)P_2 in adventitious roots of cucumber (Lanteri et al. 2008) and also at the plasma membrane of Arabidopsis roots and embryos (Tejos et al. 2014) is modulated by auxins. Since PI4P and PI(4,5)P_2 are potential PI-PLC substrates (Munnik 2014), altered levels of PI4P and PI(4,5)P_2 in *plc2* mutants might affect auxin signaling and hence embryonic development.

The two allelic T-DNA insertions in *AtPLC2* studied show a reduction in the genetic transmission through the female gametophyte. However, *plc2-2/PLC2* also shows an embryo lethal phenotype and a reduction in the genetic transmission through male gametophytes. These different terminal phenotypes observed might arise from the particular insertion site of the T-DNA into the gene. A similar allelic variation was already described for genes, whose expression is required early for normal gametogenesis as well for embryogenesis progression (Pagnussat et al. 2005; Muralla et al. 2011). In addition, these divergences might account for different levels of maternal PLC2 present in the different ecotypes.

While we were conducting our research, two papers on *PLC2* mutants appeared. Kanehara et al. (2015) used the *plc2-1* allele, while Li et al. (2015) reported a study for the *plc2-2* allele. To our surprise, Kanehara and co-workers (2015) obtained *plc2-1* homozygous mutant plants in Ws background and were able to maintain the line throughout different generations, which suggest that the plants have normal gametophyte and embryo development. The only phenotype that the authors described are that seedlings of the knock-out mutant showed increased amounts of PI4P and PI(4,5)P_2 and slight root growth retardation with hypersensitivity to ER stress (Kanehara et al. 2015). As reported in this paper, we were not able to find any *plc2-1* knock-out mutants, since the lack of *PLC2* in Ws showed a severe problem in female gametophyte development.

In contrast, our results and those reported by Li et al. (2015) showed for the independent insertion allele *plc2-2* in the Col-0 background that disruption of *PLC2* led to sterility. Self-pollinated *plc2-2/PLC2* plants show embryos with aberrant development at the pre- and globular stage and aborted seeds (around 15% reported by Li et al. (2015) and 25% in this paper). A low proportion of *plc2-2* knock-out plants was found in the progeny of self-pollinated hemizygous plants (between 4% reported by Li et al. (2015) and 0.8% in this paper). These mutants presented abnormal flower and embryo-sac development, and were not able to produce seeds. Li et al. (2015) showed that *plc2-2* knock-out plants presented normal sporophytic development, and altered male- and female-gametophyte development associated with increased auxin concentrations in the reproductive organs. However, in our hands, these mutant plants were smaller than WT plants and showed a delay in growth. Since a low proportion of individuals somehow bypassed this lethality by an unknown mechanism (which might compensate the lack of *PLC2*) and the knock-out mutants presented an altered phenotype in the sporophyte, we used hemizygous plants to study gametophyte development. Reciprocal crosses between WT and *plc2-2/PLC2* showed that both gametophytes were defective (Table S2), which might explain why the ratio of kanamycin resistant to sensitive plants was below 2:1 (albeit not significantly different) in the progeny of self-pollinated *plc2-2/PLC2* plants. As the results obtained by Li et al. (2015) indicated a problem in the transmission (only) through the male gametophyte, we further study pollen germination in *plc2-2* allele mutants. When compared to WT pollen that had an in vitro germination ratio near 70%, only 50% of the *PLC2/plc2-2* pollen grains tested were able to germinate. *plc2-2/plc2-2* pollen

grains did not germinate in vitro. However, grains were able to germinate *in planta*, explaining the fact that we were able to obtain *plc2-2/plc2-2* homozygous plants. Thus, PLC2 activity is essential for pollen germination in vitro. PLC2 requirement seems to be bypassed when pollen germinates in vivo, suggesting that the stigma is providing a downstream signal and thus promoting pollen germination. Altogether, our results point out to critical steps during reproductive development in which PLC2 is essential.

In animal cells, the levels of the nuclear PI-PLC substrates and products affect cell-cycle progression and differentiation (reviewed Cocco et al. 2009). In addition, the nuclear sperm-specific PI-PLCζ isozyme triggers Ca^{2+} oscillations in the egg, which is essential for egg activation, fertilization, and embryo development. These oscillations are cell-cycle dependent (reviewed Faenza et al. 2013). Changes in phosphoinositides during the cell cycle and a requirement for inositol for cell-cycle progression have also been documented in plants (reviewed in Dicke et al. 2012). A similar scenario could take place during female gametogenesis and embryo development, and PLC2 might somehow regulate those processes.

The lack of PLC2 may cause an accumulation or miss localization of the substrates, PI4P and/or $PI(4,5)P_2$. During cytokinesis of tobacco BY-2 cells, $PI(4,5)P_2$ has been found to lead the expanding cell plate, while PI4P was localized there right from the start (van Leeuwen et al. 2007; Vermeer et al. 2009). PI-PLC controlled the levels and distribution of $PI(4,5)P_2$, regulating pollen-tube growth (Dowd et al. 2006; Heilmann and Ischebeck 2016). PI-PLC has an active role to create and maintain a $PI(4,5)P_2$ gradient in the pollen tip, between the apical and lateral membranes, and this gradient is necessary for polarized growth. Future experiments using PI4P and $PI(4,5)P_2$ lipid biosensors expressed specifically in the female gametophyte and during embryo development will hopefully unravel the distribution of these lipids in WT as well as in *plc2* mutants.

In addition to removing PI4P and $PI(4,5)P_2$, PLC2 is expected to locally generate diacylglycerol and IP_2 and IP_3. Further phosphorylation of these products can generate PA and diacylglycerol pyrophosphate (DGPP) in addition to various inositol polyphosphates, which have all being implicated in signaling (reviewed in Munnik and Vermeer 2010). For example, IP_6 can increase intracellular Ca^{2+} (Lemtiri-Chlieh et al. 2003) and seems to be structurally required for TIR1 function (Tan et al. 2007). Genetic evidence suggests crosstalk of inositol polyphosphate and auxin signaling. For instance, several inositolphosphate 5-phosphatase mutants display altered auxin levels and responses (Carland and Nelson 2004; Chen et al. 2008; Wang et al. 2009; Lin et al. 2012). In addition, Ca^{2+} and PA are activators of the NADPH oxidase activity in plants (Zhang et al. 2009). Reactive oxygen species (ROS) and cytoplasmic Ca^{2+} oscillation have been detected in synergid cells upon pollen-tube arrival (Duan et al. 2010; Iwano et al. 2012). Since we observed expression of PLC2 at one of the synergid cells upon fertilization, we can envisage that PLC2 might contribute to Ca^{2+} and NADPH oxidase activation during fertilization. Duan et al. (2010) identified the receptor-like kinase FERONIA (FER), which is responsible for RAC/ROP (Rho-related small GTPase) signaling and targets NADPH oxidase to accumulate ROS for root hair elongation. FER is a well-known regulator of female fertility that controls the levels of ROS to induce pollen-tube rupture and sperm cells release in the embryo sac in a Ca^{2+}-dependent process (Huck et al. 2003; Duan et al. 2014).

In summary, a clear role for PLC2 in male and female gametogenesis is emerging as well as during early embryonic development. Particularly, PLC2 is required for normal mitotic divisions, probably by regulating cytoskeletal dynamics. The molecular basis underlying this process is the subject of current and future work, which will shed light on how the PLC2-signaling network is operating in reproductive tissues.

Author contribution statement AML and GCP conceived the original screening and research plans; AML, CGP, and JMD supervised the experiments; LDF performed most of the experiments; JMD, RvW, and RT provided technical assistance to LDF; LDF, AML, and GCP designed the experiments and analyzed the data; AML and GCP conceived the project and wrote the article with contributions of all the authors; LL and TM supervised and complemented the writing.

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